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Patent application No.: PA 2002 02001

Date of filing: 23 December 2002

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Title: Indretning og fremgangsmåde til bestemmelse af Embryorespiration, og til styring af oxygenpartialtrykket

IPC: G 01 N 33/50; C 12 M 1/34

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26 January 2004

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PATENT- OG VAREMÆRKESTYRELSEN

TITLE: DEVICE AND METHOD FOR MEASURING EMBRYO RESPIRATION, AND FOR CONTROLLING OXYGEN PARTIAL PRESSURE.

FIELD OF INVENTION

The present invention relates to methods and devices for non-invasive and non-disturbing measurements of embryo respiration rates and to a method and device for controlling oxygen partial pressure at the level of the embryo, as well as to a use of an oxygen partial pressure determination, in stagnant growth medium comprising at least one embryo, for selecting the most viable embryo(s).

BACKGROUND OF THE INVENTION

Use of Embryo Transfer (ET) techniques, such as IVF (In Vitro Fertilization) and related techniques, involves in vitro culturing of the developing embryo for a period of days before re-implantation of selected embryos. Even with the ideal growth conditions, selection criteria are needed as a tool to choose the most viable embryos for re-implantation. The viability of an embryo is an important parameter in order to determine the embryos suitability for transfer. At present, there are no objective means applicable on a practical level, which can serve to assess the viability of the embryo following manipulation. In practice, embryo evaluation is limited to a more or less subjective grading based on morphological criteria.

The respiration rate of the embryo may prove a good candidate for an objective viability indicator. It has previously been demonstrated that the respiration rate of bovine, murine and human embryos (expressed as oxygen consumption) is a usable indicator of embryo viability (See Shiko et al. 2001. Oxygen consumption of single bovine embryos probed by scanning electrochemical microscopy. Anal. Chem 73:3751-3758 or Trimarchi et al. 2000. Oxidative phosphorylation dependent and –independent oxygen consumption by individual preimplantation mouse embryos. Biology of reproduction 62: 1866-1874 or Overström EW et al. 1992. Viability and oxidative metabolism of the bovine blastocyst. Theriogenology 37(1): 269 or Magnusson C et al. 1986. Oxygen consumption by human oocytes and blastocysts grown in vitro. Human Reproduction 1: 183-184). These studies dem-

onstrated that a certain (high) respiration rate is correlated to an improved development such as improved in vitro development (expressed by increased blastocyst rate) or increased pregnancy rates.

A number of methods for determination of embryo respiration are known. Mills and Brinster (See Mills and Brinster 1967. Oxygen consumption of preimplantation mouse embryos. Exp. Cell. Res., 47: 337-344) describe a method using the Cartesian diver technique on batches of mouse embryos, which measures the volume change of an oxygen gas bubble in direct contact with the growth medium of the embryos.

Magnusson et al. 1986 (Oxygen consumption by human oocytes and blastocytes grown in vitro. Human Reproduction 1, 183-184) and later Houghton et al. 1996 (Oxygen consumption and energy metabolism of the early mouse embryo. Molecular reproduction and development 44:476-485) describe a method which is capable of measuring oxygen consumption of individual embryos using a sensitive micro-spectrophotometric technique, where embryos are placed in small sealed chambers and the oxygen consumption is estimated as a decrease in oxygen partial pressure, monitored as an absorbance change of a substance which optical absorbance is sensitive to the presence of oxygen. Due to the extensive handling of the embryo in and out of sealed chambers, the measurements are disturbing to the embryo as well as time consuming.

Another technique has been described in which embryos are fixed on a thin capillary and oxygen concentration gradients are measured with very precisely positioned oscillating oxygen microelectrodes under the assumption of spherical diffusion (See Shiko et al., 2001. Oxygen consumption of single bovine embryoes probed by scanning electrochemical microscopy. Anal. Chem 73: 3751-3758, or Trimarchi JR, et al., 2000. Oxidative phosphorylation dependent and independent oxygen consumption by individual preimplantation mouse embryos. Biology of reproduction 62: 1866-1874). These techniques are characterized by relatively com-

plicated experimental designs which are demanding to operate, and results in significant disturbance of the embryo. It is furthermore time consuming to perform the measurement and the presumptions for the method are demanding to fulfill.

In general, the above-mentioned studies and related studies to measure individual embryo respiration suffer from being complicated, disturbing to the embryo and time consuming, and it is therefore not very likely that such methods will be applied routinely for monitoring individual respiration rates of embryos in cultures in vitro. A need therefore still exists for a fast, simple and non-disturbing method and device for measuring individual embryo respiration rates, as a measure for the embryo viability. This need is widely expressed by researchers and practitioners of embryo transfer techniques involving in vitro culture of embryos. Overstöm 1996 (See Overström EW 1996, In vitro assessment of embryo viability. Theriogenology 45:3-16) compiles in a literature review the demand for a simple and objective method for determination of individual embryo respiration, as an expression of embryo viability. As the embryo in vitro techniques becomes more sophisticated, including ICSI (Intra Cytoplasmic Sperm Injection), cloning and freeze cycles, this demand is expected to become even more pronounced. Within the field of human infertility treatment, it has become necessary to focus on single embryo transfer to avoid unwanted multiple pregnancies, which are the consequence of multiple embryo transfer. Single embryo transfer, however, calls for a close viability assessment in order be able to select the best embryos and thereby increase the probability of a successful pregnancy, which again stresses the need for simple and objective viability indicators applicable on a routine level. A new method should preferably contain the following key elements as outlined by Overström 1996 (see In vitro assessment of embryo viability. Theriogenology 45:3-16 1996).

-The ability to make simultaneous objective measurements of multiple individual embryos.

-The sensitivity and resolution to measure an individual embryo/oocyte.

-Rapid evaluation (~30 min or less).

-Viability test must be non-perturbating and ideally non-invasive.
-Technically simple and user friendly.
-Affordable.

In addition to the expressed need for a method and device for respiration measurements applicable on a routine level, in vitro culture of embryos suffers from an insufficient control of the oxygen partial pressures as experienced by the developing embryo. In vitro culture of embryos is often carried out in incubators with regulated atmosphere (temperature, relative humidity and gas composition). Atmospheric air contains 21% oxygen (210 hPa partial pressure), but in vivo (oviduct and uterus) oxygen tensions are considered to be around 5-10% oxygen (50-100hPa) saturation. It is therefore not surprising that, in general, embryo development is better under a 5-10% atmosphere than under air. Lim et al. and Thompson et al. (See: Lim et al. 1999 Development of in vitro bovine embryos cultured in 5% CO_2 in air or 5% O_2 , 5% CO_2 and 90% N_2. Human reproduction 7(4):558-562 or Thompson JGE et al. 1990 Effect of oxygen concentration on in vitro development of preimplantation sheep and cattle embryos. J. Reprod. Fert. 89, 573-578) and others previously demonstrated the positive effect of reduced oxygen partial pressure on the mammalian embryo development. Embryos are therefore in some cases cultured under a reduced oxygen atmosphere, e.g. 5% saturation. It is however insufficient to control the embryos exposure to oxygen by alone controlling the atmosphere above the medium. The medium is typically oxygen saturated (21%) when initiating the in vitro culture, and the equilibration time between the medium and the overlaying gas atmosphere can, depending on the in vitro growth system, be as long as 12-24 hours, such that the embryo for a significant period of the in vitro culture, will experience oxygen partial pressure significantly exceeding what at present is considered the optimal (5-10%). The final steady state partial pressure at the surface of the embryo will however be lower than that of the above atmosphere, e.g. 5%, due to the steady state oxygen partial pressure gradient from the bulk medium towards the embryo, arising as a result of the embryo respiration.

A need therefore still exists for a simple and fast (<1 h) method to regulate the oxygen partial pressure as experienced by the developing embryo during in vitro culture.

SUMMARY OF THE INVENTION

It is the object of the present invention to provide a method and device for measurement of individual embryo respiration and a method and a device for controlling oxygen partial pressure at the level of the developing embryo.

It is a further object of the present invention to provide a method for respiration measurements during in vitro culture with a response time not exceeding few seconds.

It is also a further object of the present invention to provide a simple device for a rapid (~30-120 min) adjustment of the oxygen partial pressure as experienced by the developing embryo during in vitro culture.

In a first aspect the present invention relates to a device for non-invasive measurement of the individual respiration rate of at least one embryo, which device comprises:

a) at least one compartment of a gas impermeable material with at least one opening;

b) a gas permeable layer in the bottom of the at least one compartment; and

c) a detector that measures the partial pressure of a gas inside the compartment.

In a second aspect the present invention relates to a non-invasive method for measuring the respiration rate of at least one individual embryo and/or for controlling the oxygen partial pressure experienced by the embryo comprising:

a) providing at least one compartment of a gas impermeable material with at least one opening;

b) incubating at least one embryo in a suitable growth medium in the said compartment under conditions where the growth medium in the compartment is stagnant and oxygen is supplied from the surroundings to the embryo via the opening by diffusion through the stagnant medium inside the compartment; and

c) determining the respiration rate of the embryo by measuring a gas partial pressure at a position inside the compartment.

In a third aspect the present invention relates to a use of an oxygen partial pressure determination, in stagnant growth medium comprising at least one embryo, for selecting the most viable embryo(s), wherein the oxygen partial pressure at the position of the embryo is dependent on the supply rate of oxygen from the surroundings to the embryo via an opening by diffusion through the stagnant medium, and said oxygen partial pressure determination is performed without causing any change in growth conditions experienced by the embryo.

In a fourth aspect the present invention relates to a method of controlling the oxygen partial pressure experienced by an individual embryo, wherein the said embryo is grown in stagnant medium in a device according to the invention.

In a fifth aspect the present invention relates to a use of an oxygen partial pressure determination, in stagnant growth medium inside a compartment comprising at least one embryo, for selecting the most viable embryo(s), wherein the oxygen partial pressure at the position of the embryo is dependent on the supply of oxygen from the surroundings to the embryo via an opening by diffusion through the stagnant medium, and said oxygen partial pressure determination is performed without causing any change in growth conditions experienced by the embryo.

BRIEF DESCRIPTION OF DRAWINGS

The invention will now be described by way of exemplifying embodiments hereof and with reference to the accompanying drawings in which

Figure 1 is a cross section of a first embodiment of a diffusion compartment with an oxygen sensor at the bottom, according to the present invention. The theoretical steady state oxygen gradient is shown in a graph next to the drawing.

Figure 2 is a cross section of a compartment with an insert to adjust the internal transverse dimension of the first embodiment.

Figure 3 is a cross section of another embodiment of the present invention comprising a diffusion compartment with an adjustable bottom.

Figure 4 is an example of the steady state oxygen gradient measured inside a cylindrical diffusion compartment, where an embryo is cultured at the bottom. The linear part of the gradient in figure 4 corresponds to a section of the solid part of the line in the theoretical graph in figure 1. The unit on the x-axis is hPa and the unit on y-axis is µm. The position of the opening of the compartment 5 in relation to the gradient is marked with the vertical line.

Figure 5A is another embodiment of the said diffusion compartment where the diffusion compartment is completely open and the oxygen gradient is recorded in two dimensions around the embryo. 5B shows a cross section of the bottom at the level of the embryo. 5C shows an image (top or bottom view) as seen from the CCD camera, where the luminescence intensity of the luminophore around each individual embryo is visualized in grey tones.

Figure 6A is an example of the steady state oxygen gradient measured towards an embryo along the plane bottom of an open compartment as illustrated in figure 5. Figure 6B is a plot to illustrate how the actual gradient fit to a theoretically ideal spherical gradient. If the plot is linear, the assumption of a spherical diffusion system is fulfilled.

DETAILED DESCRIPTION OF THE INVENTION

Prior to a discussion of the detailed embodiments of the invention, a definition of specific terms related to the main aspects of the invention is provided.

Oxygen partial pressure: The pressure that oxygen as a single component would exert. The total gas pressure is the sum of individual gas pressures. Under normal atmospheric conditions the total actual gas pressure will be close to 1 atm or 1000 hPa. Atmospheric oxygen partial pressure is approximately 21% or 210 hPa. The oxygen concentration C is equal to the oxygen partial pressure P multiplied by the oxygen solubility S, ($C = PS$), where the solubility S is a function of temperature, salinity and total gas pressure.

Noninvasive method: A method, which without any destructive disturbance, or without requiring insertion of an instrument or device through the skin or body orifice can measure a parameter related to a body of interest.

Respiration rate: Most living organisms, including developing embryos, consume oxygen in their energy metabolism, by a process called respiration. The oxygen consumption rate of a respiring organism is also named the respiration rate. The respiration rate of human embryos has previously been determined to be in the range 0.34 – 0.53 nl O_2 embryo^{-1} h^{-1}, but embryo respiration rates can vary considerably during the development from oocyte over morula to the blastocyst stage (See Magnusson C et al. 1986. Oxygen consumption by human oocytes and blastocysts grown in vitro. Human Reproduction 1: 183-184). Bovine embryos will typically have respiration rates in the range from 1-8 nl O_2 embryo^{-1} h^{-1}.

Response time: The time from initiating a measurement until a response or signal adequate for the measurement is obtained, and the measurement can be considered successful.

Amperometric oxygen sensor: A Clarck type electrochemical sensor with a gold cathode polarized against an internal reference, where oxygen is reduced on the cathode surface. A current meter converts the resulting reduction current to a signal.

Bulk medium: Medium in the surroundings outside the compartment or at a distance from the embryo such that the respiration of the embryo does not influence the oxygen partial pressure of the bulk medium.

Medium: Liquid growth substance for the embryo, such as a fluid growth substance, preferably a liquid growth substance.

Membrane inlet mass spectrometry (MIMS):. A technique for measuring oxygen and other dissolved gassed, based on a tube equipped with a gas permeable membrane, connected to the inlet of a mass spectrometer. Due to a vacuum (applied by the mass spectrometer) inside the tube, gas enters the mass spectrometer through the gas permeable membrane. The mass and composition of the gas is subsequently determined by the mass spectrometer.

Microspectrophotometric technique: A technique for measuring oxygen based on an increase or decrease in absorbance at 435 nm, reflecting dissociation of oxyhemoglobin due to a decrease or increase in oxygen partial pressure. Other oxygen binding molecules with other absorption characteristics may be used.

Diffusion: The process whereby particles of liquids, gases, or solids intermingle as the result of random molecular motions caused by thermal agitation, resulting in a net transport of dissolved substances from a region of higher to one of lower concentration.

Diffusion compartment: A space or compartment of defined internal dimension with a defined opening towards an exterior environment. The liquid based material in-

side the diffusion compartment is stagnant, primarily due to frictional forces between the liquid and the compartment wall. The diffusion compartment is also referred to as the "compartment" in the device and method of the present invention.

Stagnant liquid: A liquid without any flow, turbulence or movement. Transport of dissolved substances primarily takes place by diffusion.

Steady state: A situation where consumption and transport is in equilibrium such that gas partial pressure, or concentration gradients of dissolved substances, are stable and no partial pressure change or concentration change takes place.

Optical oxygen sensing: A measuring principle based on the ability of oxygen to act as a dynamic luminescence quencher of a luminophore. The luminophore is excited by defined wavelengths, and luminescence is emitted by the luminescent indicator as a function of oxygen concentration. In the presence of O_2 the intensity and the decay time of the luminescence decreases in a predictable way due to the quenching process. Optical oxygen sensing in two dimensions can be based on luminescence lifetime imaging, which in some cases is advantageous over luminescence intensity imaging. Oxygen luminophores can be made of Ruthenium(II)-tris-4,7-diphenyl-1,10-phenatroline per chlorate (Rudpp) immobilised in a polystyrene matrix, Ruthenium (II) tris-1,7-diphenyl-1,10-phenanthroline chloride, Ruthenium(II)-tris(bipyridyl) complex, Tris (2,2'-bipyridyl di-chloro-ruthenium) hexahydrate, Ru(bpy), Platinum (II)-octa-ethyl-porphyrin in polystyrene, Platinum (II)-octa-ethyl-porphyrin in poly(methyl-methacrylate), Platinum (II)-octa-ethyl-keto-porphyrin in polystyrene, Platinum (II)-octa-ethyl-keto-porphyrin, Palladium (II)-octa-ethyl-porphyrin in polystyrene, Platinum 1,2 ene-dithiolates (class of compounds), or other luminophores suitable for oxygen detection in the present invention.

Luminescence: Production of light.

The present invention relates to establishing the respiration rate of an individual embryo in a fast and convenient way without causing perturbation to the embryo. In the method and device for individual embryo respiration and oxygen partial pressure control, according to the invention, an embryo is placed or cultured in a compartment with defined dimensions made from a gas impermeable material having at least one opening, which opening is gas permeable. The opening could be covered by a gas permeable membrane. In one particular embodiment the side walls and the bottom are made of a gas impermeable material. The compartment comprising the embryo in a suitable growth medium is in open connection with an atmosphere of known gaseous composition, and controlled temperature and humidity, directly via the opening or through a larger volume of medium outside the compartment. Oxygen and other dissolved substances are supplied to the embryo directly from the atmosphere or via the larger volume of medium in equilibrium with the atmosphere, through the defined diffusion compartment by diffusion through the stagnant medium inside the compartment. The oxygen partial pressure outside the compartment will in both cases be known. Either the composition of the atmosphere is known or the bulk medium will be in equilibrium with the atmosphere of known composition.

The device is designed to keep the medium inside the compartment stagnant, such that transport of substances dissolved in the medium can alone take place by diffusion. When the embryo is in the stagnant medium inside the compartment, the oxygen partial pressure close to the embryo will, due to the oxygen consumption of the embryo, be reduced compared to the oxygen partial pressure outside the compartment. In a steady state situation, the supply of oxygen equals the consumption and the oxygen partial pressure gradient towards the embryo will be stable. The steepness of the gradient from the opening of the diffusion space or at a distance from the embryo, towards the embryo, is thus a measure of the embryo oxygen consumption (respiration). The respiration rate of the embryo is measured by determining the oxygen partial pressure or concentration at a position inside the compartment. One measurement will be sufficient for determining the respiration

rate under the above described conditions. The bulk medium outside the compartment does not have to be stagnant.

Depending on the shape of the compartment, the steady state partial pressure gradient from the opening of the diffusion compartment towards the embryo can be linear, semi spherical, a combination of both or another form. If the diffusion compartment is designed such that the shape of the partial pressure gradient inside the diffusion compartment is predictable (e.g. linear) and can be described mathematically, and the partial pressure outside the compartment is known, the absolute embryo consumption can be estimated by a single oxygen measurement along the partial pressure gradient towards the embryo. Taking figure 1 as an example this measurement should preferably be performed at a position inside the compartment in which the oxygen gradient is linear as illustrated. The oxygen partial pressure measurement can be in one, two or three dimensions depending on the detection principle. If the nature of the gradient in the compartment, caused by the embryo respiration, can not be described fully, or the internal dimensions of the compartment are not well defined, the device can be calibrated by using artificial embryos with a known oxygen consumption. Artificial embryos for calibration can be small spherical particles with the diameter of an embryo, 50-200 µm, made of an oxygen consuming material (antioxidant), like vitamin C, E, A, carotenoids, selenium, titanium chloride, dithionite, ferrous sulfides, embedded in a stable auxiliary compound like starch, or coated onto inert spherical bodies like glass beads.

In case the oxygen partial pressure gradient inside the compartment is not in steady state and still develops, which may be the case shortly after the embryo is placed in the compartment, the respiration rate may still be determined by investigating the change of the oxygen partial pressure gradient inside the compartment per time unit. The steady state gradient can in other words be modeled mathematically from a series of non steady state gradients over time.

It is also an aspect of to the present invention that the said diffusion compartment will reduce the oxygen partial pressure experienced by the embryo and function as a means for reducing the developing embryos exposure to oxygen to a desirable level. For this particular purpose, the diffusion compartment need not necessarily to be equipped with a means for detection of oxygen if judged unnecessary to estimate the exact partial pressure as experienced by the embryo.

In one embodiment the invention relates to a method of controlling the oxygen partial pressure experienced by an individual embryo, wherein the said embryo is grown in stagnant medium in a device according to the invention.

According to the invention it is furthermore possible to carry out measurements of embryo consumption or production of other substances than oxygen of which a partial pressure or concentration gradient can be established in the said diffusion compartment. In addition to O_2 luminophores, fluorescent indicators for CO_2, Ca^{2+}, and pH are available and can be applied in the present invention.

In a further embodiment of the present invention the partial pressure determined at a position inside the compartment is the CO_2 partial pressure.

The said diffusion compartment with or without an oxygen sensor could be used as a growth chamber throughout the in vitro culture period of the embryo.

The external dimensions and shape of the diffusion compartment with an oxygen sensor, can be varied to fit into any container system, making it adaptable to existing in vitro culture systems and measuring systems in general.

Oxygen detection can be based on optical sensing (see definitions) with immobilized luminophore, optical sensing with luminophores dissolved in the medium, microspectrophotometric techniques, electrochemically based oxygen sensors including Clack type oxygen sensors, MIMS technology (membrane inlet mass spec-

trometry) or any other means of detection conceivable by a person skilled in that art. In a particular embodiment of the invention the oxygen partial pressure or concentration is determined using an immobilized luminophore layer and recording the luminescence with a luminescence reader or a camera such as a CCD-camera.

In one embodiment, the oxygen sensor can be electrochemical or any other detection principle for oxygen.

The oxygen concentration determination is in a particular embodiment performed in the bottom of the compartment an in another embodiment the means for oxygen determination is placed at the bottom of the compartment underneath a gas permeable layer and the at least one embryo is resting on the gas permeable layer.

In one embodiment the oxygen partial pressure is determined with a Clark type electrochemical oxygen micro sensor with a tip diameter not exceeding the transverse diameter of the compartment, placed at the bottom of the compartment with the sensor tip penetrating the oxygen impermeable bottom wall (4) of the compartment. The sensor tip is separated from the embryo by an oxygen permeable layer (6). The oxygen sensor should be of a design such that the analyte (oxygen) consumption of the sensor does not exceed a negligible fraction, such as 1%, of the embryo respiration rate, such that the oxygen partial pressure inside the compartment gradient is not disturbed by the measuring activity of the said sensor.

In another embodiment the Clark type oxygen sensor is replaced by a MIMS fiber penetrating the oxygen impermeable bottom wall (4) of the compartment. The MIMS fiber tip is separated from the embryo by an gas permeable layer (6). The MIMS fiber should be of a such design that the analyte (any gas which can migrate through the MIMS fiber membrane and is detectable on a mass spectrometer) consumption of the sensor does not exceed a negligible fraction, such as 1%, of the embryo consumption or production rate, such that the gradient of a particular

gas inside the compartment gradient is not disturbed by the measuring activity of the said MIMS fiber.

In yet another embodiment, the oxygen partial pressure gradient inside the compartment is determined by adding oxyhaemoglobin, or another molecule with an oxygen dependant absorption characteristic, to the growth medium and measuring the absorbance gradient at 435 nm, or another suitable wavelength, through transparent sidewalls of the compartment, and thereby determining the oxygen distribution in the compartment.

In the following a number of different embodiments of the present invention will be described with reference to the accompanying drawings, but it is to be understood that these embodiments only constitute examples of the general inventive idea, and that other embodiments may be conceivable by a person skilled in the art.

The embodiment of the invention shown in figure 1 for measuring embryo respiration illustrates a longitudinal compartment 1 open in one end. The bottom of the compartment, which could be cylindrical, consists of a gas permeable substance 6 on top of a transparent oxygen sensitive luminophore 3. The bottom wall 4 of the diffusion compartment is made of a transparent material which allows visual inspection of the embryo under magnification. The bottom wall 4 is made of a gas impermeable material like glass or plastic, such that the only supply of oxygen is through the opening of the compartment 5. Oxygen partial pressure, in the luminophore layer 3 at the bottom of the compartment, is measured by means of an external luminescence reader by recording luminescence from the oxygen luminophore 3 at the bottom of the compartment, through the transparent bottom wall 4. The surroundings 7 which in one embodiment could be bulk medium is in equilibrium with an atmosphere of known or unknown gaseous composition. The device accommodates a single or several embryos 8 placed on the gas permeable substance 6, which substance in one embodiment is silicone, on top of a transparent oxygen sensitive luminophore 3. The gas permeable substance 6 can be a silicone

compound, a Teflon fluoropolymere, a plastic compound like polyethylene, polypropylene or neoprene, a permeable matrix or porous material based on another chemically inert material like glass, ceramics or minerals, glass or mineral fibers or a precious metal like gold or platinum.

The functional principle of the invention is that the embryo's consumption of oxygen reduces the oxygen partial pressure at the oxygen sensor (luminophore) 3 compared to the oxygen partial pressure in the bulk medium/surroundings 7. The oxygen partial pressure gradient 9 will in steady state be stable and not subject to change as long as the embryo's oxygen consumption is constant. In the present embodiment comprising a longitudinal cylindrical diffusion compartment, the oxygen partial pressure gradient will be linear as indicated in figure 1. Real experimental data are shown in figure 4. The oxygen consumption by the embryo will therefore be determined as the difference between the oxygen partial pressure at the bottom 4 and the opening 5 of the said diffusion compartment 1 using Ficks 1. law of diffusion (equation I),

$$J = -D\frac{dC}{dx} \qquad (I)$$

assuming a linear decrease (see the theoretical graph 9) of oxygen from the opening towards the embryo 8 at the bottom, where J is the flux of oxygen, which in steady state equals the consumption of the embryo, D is a known diffusion coefficient of the medium and dC/dX is the oxygen gradient. The gradient dC/dX is the difference in oxygen partial pressure between the defined atmosphere or medium at the opening 5 of the compartment and the bottom of the compartment at the level of the embryo 8. The use in the present embodiment of an optical oxygen luminophore 3, covering the bottom 4 of the compartment 1, will integrate the oxygen signal over the total bottom area. Horizontal oxygen gradients at the level of the embryo, arising from an unevenly distributed oxygen consumption related to the embryo, will be averaged, as if the consumption was evenly distributed over

the bottom area, such that the exact placement of the embryo becomes irrelevant for the respiration estimation.

The respiration rate of an individual embryo can thus be determined by a single oxygen partial pressure measurement, performed from the outside of the diffusion compartment without perturbing the embryo, by oxygen detector means immobilized inside the compartment. The measurement can be performed within a few seconds without any disturbance of the embryo. Depending on the detector means, the measurement can be performed inside the incubator, which could e.g. be an incubator or a warm room, or the measurement can be performed within a very short time outside the incubator, such that growth conditions experienced by the embryo is not significantly affected.

By using an array of compartments of the present invention, multiple embryos can be scanned for individual respiration rates simultaneously. In one embodiment the at least one compartment comprises at least 5 compartments, particularly at least 10 compartments, more particularly at least 24 compartments, and even more particularly at least 96 compartments.

In a further embodiment individual embryos are grown in separate compartments and in a still further embodiment each compartment comprises more than one embryo.

Figure 2 shows an insert 10 inside the first embodiment, which serves to adjust the transverse dimension A of the longitudinal compartment 1. By narrowing or enlarging the transverse dimension the capacity of the diffusion compartment to transport dissolved substances by diffusion can be increased or reduced. The transport capacity of the diffusion compartment determines the steady state oxygen partial pressure at the position of the embryo.

In one embodiment of the invention the oxygen partial pressure at the position of the embryo is controlled by adjusting the volume of the compartment 1. This can be done in several ways, e.g. by adjusting the position of a adjustable bottom 11 (see fig. 3), by decreasing or increasing the medium level inside the compartment, or by introducing an insert 10 (see fig. 2) into the compartment, which will reduce the transverse dimension A.

In this respect the dimensions of the compartment relative to the size of the embryo is important. Typically the transverse dimensions of the compartment 1 is less than 2.5 mm, particularly less than 1.5 mm, more particularly less than 500 µm, such as less than 250 µm. The transverse dimension B of the insert is typically less than less than 1.5 mm, particularly less than 1.0 mm, more particularly less than 500 µm, and even more particularly less than 300 µm. The bottom part of the compartment below the insert could in one embodiment be maintained with an transverse dimension of less than 2.5 mm.

The longitudinal dimension of the compartment is in one embodiment between 2 to 25 mm, particularly between 3 to 15 mm.

The thickness of the gas permeable layer 6 is in one embodiment at least 100 µm, particularly at least 300 µm, and more particularly at least 900 µm. The thickness of the gas permeable layer should preferably be about twice or more the diameter of the embryo, which for mammalian embryos typically is between 30-400 µm dependent on the developmental stage and species.

The medium level in the compartment can be varied in a controlled way by adding or removing a defined quantity of medium. The functional principle of this relates to increasing or decreasing the distance in the stagnant medium through which the oxygen has to diffuse, corresponding to altering the dimensions of the effective diffusion compartment 1 and thus controlling the transport of oxygen from the atmosphere above the medium of constant composition, to the embryo 8. The respiration

rate of the embryo can be determined with the option of adjusting the medium level and thus the oxygen partial pressure as experienced by the embryo to a desired level, at any respiration rate.

Figure 3 shows another embodiment of the present invention. Elements identical with elements of the first embodiment shown in Figure 1 are designated by the same reference numbers as on figure 1. The embodiment consists of a compartment 1, e.g. a cylindrical compartment, with a opening 5 in one end, but with a moveable or adjustable bottom 11 with a gas permeable layer 6 on top of an oxygen sensitive luminophore 3. The bottom wall 11 is sealed against the compartment wall 2 such that the seal is gas impermeable.

The dimension of the compartment in the second embodiment of the present invention can due to the moveable bottom 11 be altered in a controlled way either increasing or decreasing the diffusion length of oxygen from the opening of the compartment 5 to the embryo 8. By increasing or decreasing the length of the compartment 1, the steady state oxygen partial pressure at the level of the embryo 8 can be either decreased or increased to reach a desired oxygen partial pressure, without affecting the possibility of performing a respiration estimation. The respiration rate of the embryo can in this way be determined with the option of adjusting the oxygen partial pressure as experienced by the embryo to a desired level, at any respiration rate.

Figure 5 is yet another embodiment of the present invention where the complete volume of the incubation medium within a growth dish defines the compartment 1, which is then much larger than in the other embodiments of the present invention. The bottom 4 of the growth dish is transparent and covered with a luminophore 3 on top of which is placed one or several embryos 8 at a distance from each other, large enough, typically more than 2 mm, to avoid overlap of partial pressure gradients among the embryos. The functional principle of the present embodiment is that oxygen is supplied to the embryo from the surrounding medium in contact with

the atmosphere outside the compartment above the embryo as illustrated in figure 5 B. When the compartment is very large relative to the embryo, the resulting oxygen gradient towards the embryo will be spherical as illustrated by the oxygen partial pressure iso-lines 13 in figure 5B, and real data from Figure 6 The growth dish constituting the diffusion compartment is placed on a CCD camera 12 which by optical oxygen sensing resolves the horizontal distribution of oxygen in the luminophore 3 in two dimensions. The signal from the CCD camera 12 corresponding to the area around each embryo 8 will thus become a measure of the individual embryo respiration. The effect is shown in figure 5C, which shows an image as seen from the CCD camera, where the luminescence intensity of the luminophore around each individual embryo is visualized in grey tones. Embryo respiration is estimated by fitting a recorded oxygen partial pressure gradient around the embryo to a theoretical model assuming ideal spherical diffusion. The gradient of oxygen towards an oxygen consuming body in a free diffusion space can be described theoretically: The concentration C at a given point r in a hollow sphere between a and b (a<r<b) can be described if the concentration at a (C1) and at b (C2) is know (Crank 1997). There is no consumption of oxygen between a and b.

$$C(r) = \frac{aC_1(b-r) + bC_2(r-a)}{r(b-a)} \quad \text{(equation II)}$$

The flux of oxygen diffusing through the spherical wall J is given by

$$J = 4\pi D \frac{ab}{b-a}(C_2 - C_1) \quad \text{(equation III)}$$

Where D is the diffusion coefficient of oxygen in the media.

The gradient is symmetrical around the oxygen consuming body and can be mirrored at any plane through the center of the body. It is hence possible to consider an oxygen consuming body, in this case an embryo, placed on a plane surface at the bottom of a large compartment (Diameter > 1cm and height more than 2mm),

as the center of a sphere, only such that the oxygen consumed by the embryo will be supplied from a half sphere. The calculated respiration rate (flux of oxygen through the spherical wall), when fitting the recorded gradient to the theoretical model, should therefore be divided by two. If the nature of the gradient in the diffusion compartment, caused by the embryo respiration, can not be described fully, the device can be calibrated by using artificial embryos with a known oxygen consumption. The embodiment is also suitable for a relative comparison of respirations rates among embryos cultured on the same compartment bottom with a 2D recording of oxygen distribution.

EXAMPLES.

Example 1

A bovine embryo was placed at the bottom of a cylindrical compartment with a diameter of 1 mm and a depth of 4 mm and cultured under an atmosphere with an oxygen partial pressure of 55 hPa. The steady state oxygen partial pressure gradient inside the compartment was measured with 100 µm intervals from the opening of the compartment towards the embryo. The time *t* (in seconds) before steady state is achieved can be approximated by the following formula,

$$t = \frac{0.45l^2}{D}$$ (From J. Crank 1995, The Mathematics of Diffusion)

where *l* is the depth of the diffusion compartment in cm and *D* is the diffusion coefficient of the medium. Steady state in a compartment with a diameter of 1 mm and a depth of 4 mm will thus be achieved after approximately 35 minutes assuming a *D* of 3.5×10^{-5}. A Clarck type oxygen micro sensor with a tip size of 10 µm, positioned with a micromanipulator, was used. The data, as shown in figure 4, show a linear gradient through the compartment. It is thus sufficient to know the oxygen partial pressure at the top and the bottom of the compartment to determine the gradient. From figure 4 it is furthermore obvious that the gradient can be determined by measuring the oxygen partial pressure at any point along the linear gradient inside the compartment, from the opening towards the embryo.

For practical purposes, and when using a luminophore layer, it is more convenient to place the means for oxygen detection in the bottom of the compartment.

Example 2

After the embryo manipulation, each individual embryo is transferred by pipette to a compartment (In vitro fertilization, cloning, thawing or another technique. See e.g.: In vitro fertilization. Kay Elder, Brian Dale, 2nd rep. Ed, Cambridge University Press (2001), for a general description of embryo manipulation techniques). The compartment is comprised within a larger frame with several compartments, such that one or several batches of embryos, from one or several humans or animals, can be contained in a single frame with multiple compartments, or groups of compartments. The frame is then incubated under desired conditions, which for human embryos typically would be 37°C, 5-21% O_2 and 5% CO_2 in N_2, 100% humidity, grown in commercially available culture medium (e.g. IVF-50 from Scandinavian IVF Science AB, Göteborg, Sweden). The medium of choice depends on the acceptance of quality control and availability of media rather than any specific type. Relatively simple balanced salt solutions for culture of embryos can be used. Earle's, Tyrode's and Hepes media have been successfully introduced. These media are available commercially as single strength or concentrated solution. The respiration measurement is performed by placing the frame in a specially designed luminescence reader, which yields a luminescence signal from the luminophore at the bottom of each individual compartment. The frame is returned to the incubator immediately after the measurements. The actual respiration rate is calculated with information about each individual compartment dimension. If the oxygen partial pressure at the position of the embryo is not within a given optimal interval, e.g. between 5-10 %, the compartment dimensions, and thus oxygen partial pressure, is adjusted e.g. with an appropriate insert.

The respiration measurement is performed as often as required during the in vitro culture period. The embryos respiration rate, typically in combination with a mor-

phological evaluation, is then used as the basis for selection of the embryos for transfer to the recipient.

Morphological evaluation in vitro is based on several features of the embryo. Such evaluation methods are subjective and depend very much on experience. The embryo is spherical and is composed of cells (blastomeres) surrounded by a gelatine-like shell, an acellular matrix known as the zona pellucida. The zona pellucida performs a variety of functions until the embryo hatches, and is a good landmark for embryo evaluation. The zona is spherical and translucent, and should be clearly distinguishable from cellular debris. The important criteria in a morphological evaluation of embryos are: (1) shape of the embryo; (2) presence of a zona pellucida; (3) size; (4) colour; (5) knowledge of the age of the embryo in relation to its developmental stage, and (6) blastomere membrane integrity. During embryonic development, blastomere numbers increase geometrically (1-2-4-8-16- etc.). Synchronous cell division is generally maintained to the 16-cell stage in embryos. After that, cell division becomes asynchronous and finally individual cells possess their own cell cycle. The cells composing the embryo should be easily identified by the 16-cell stages as spherical cells. After the 32-cell stage (morula stage), embryos undergo compaction. As a result, individual cells in the embryo are difficult to evaluate beyond this stage. Human embryos produced during infertility treatment are usually transferred to the recipient before the morula stage, whereas other mammalian embryos often are cultured experimentally to a further development stage (expanded blastocysts) before transfer to the recipient or discharge.

Example 3

Modeled semi-spherical diffusion: Figure 6A shows an oxygen profile towards a bovine embryo lying on the flat bottom of a large compartment. Figure 6B displays the same data in C(r) versus a/r, where a is the distance from the sphere center (center of embryo) to the chosen endpoint (towards the embryo) of the oxygen profile. In case the profile starts at the surface of the embryo, a is the radius of the embryo (a can be chosen also at a point distant from the embryo). The assumption

about spherical diffusion is fulfilled if the C(r) versus a/r is linear, for very large b values (when C2 is the true bulk concentration).

The flux of oxygen passing through the sphere at point a can be calculated as described previously. Figure 6B shows that the assumption of perfect spherical diffusion is not completely fulfilled in this particular case, as the line is not completely linear. The consumption estimate will hence be influenced by the choice of a, which is not the case for a perfect fit.

CLAIMS.

1. A device for non-invasive measurement of the individual respiration rate of at least one embryo, which device comprises:

a) at least one compartment (1) of a gas impermeable material with at least one opening;

b) a gas permeable layer (6) in the bottom of the at least one compartment; and

c) a detector for measuring the partial pressure of a gas inside the compartment.

2. The device according to claim 1, wherein the gas is O_2 or CO_2.

3. The device according to any of the preceding claims, wherein the opening is gas permeable.

4. The device according to any of the preceding claims, wherein the detector is an oxygen detector.

5. The device according to claim 1, wherein the gas permeable layer is placed above the detector that measures the gas partial pressure.

6. The device according to any of the preceding claims, wherein the detector for measuring the oxygen partial pressure comprises amperometric oxygen sensors, membrane inlet mass spectrometry, microspectrophotometry, or optical oxygen sensing.

7. The device according to claim 6, wherein the optical oxygen sensing is performed using an immobilized luminophore (3) placed inside the compartment, particularly in the bottom, and a detector of luminescence.

8. The device according to claim 7, wherein the luminophore comprises Ruthenium(II)-tris-4,7-diphenyl-1,10-phenatroline per chlorate (Rudpp) immobilised in a polystyrene matrix, Ruthenium (II) tris-1,7-diphenyl-1,10-phenanthroline chloride,

Ruthenium(II)-tris(bipyridyl) complex, Tris (2,2'-bipyridyl di-chloro-ruthenium) hexa-hydrate, Ru(bpy), Platinum (II)-octa-ethyl-porphyrin in polystyrene, Platinum (II)-octa-ethyl-porphyrin in poly(methyl-methacrylate), Platinum (II)-octa-ethyl-keto-porphyrin in polystyrene, Platinum (II)-octa-ethyl-keto-porphyrin, Palladium (II)-octa-ethyl-porphyrin in polystyrene, Platinum-1,2-ene-dithiolates (class of compounds).

9. The device according to claim 7, wherein the detector of luminescence is a luminescence reader or a CCD camera (12).

10. The device according to any of the preceding claims, wherein the gas permeable layer comprises silicone, Teflon fluoropolymers, plastic compounds such as polyethylene, polypropylene or neoprene.

11. The device according to any of the claims 1-9, wherein the gas permeable layer comprises permeable matrixes or porous material such as glass, ceramics, minerals, glass or mineral fibers, or precious metal such as gold or platinum.

12. The device according to claim 10, wherein the gas permeable layer comprises silicone.

13. The device according to any of the preceding claims comprising an insert (10) for the adjustment of the transverse dimension (A) of the compartment (1).

14. The device according to any of the preceding claims, wherein the bottom (11) is adjustable in order to either increase or decrease the compartment volume.

15. The device according to any of the preceding claims, wherein the transverse dimension (A) is less than 2.5 mm, particularly less than 1.5 mm, more particularly less than 500 µm, such as less than 250 µm.

16. The device according to claim 13, wherein the transverse dimension (B) of the insert is less than 1.5 mm, particularly less than 1.0 mm, more particularly less than 500 µm, even more particularly less than 300 µm.

17. The device according to any of the preceding claims wherein the longitudinal dimension of the compartment (1) is between 2 mm to 25 mm, particularly between 3 mm to 15 mm.

18. The device according to any of the preceding claims, wherein the dimension of the gas permeable layer (6) is at least 100 µm, particularly at least 300 µm, and more particularly at least 900 µm.

19. The device according to any of the preceding claims comprising at least 10, particularly at least 24, and more particularly at least 96 compartments.

20. A non-invasive method for measuring the respiration rate of at least one individual embryo and/or for controlling the oxygen partial pressure experienced by the embryo comprising:

a) providing at least one compartment(1) of a gas impermeable material with at least one opening;

b) incubating at least one embryo in a suitable growth medium in the said compartment under conditions where the growth medium in the compartment is stagnant and oxygen is supplied from the surroundings (7) to the embryo via the opening (5) by diffusion through the stagnant medium inside the compartment (1); and

c) determining the respiration rate of the embryo by measuring a gas partial pressure at a position inside the compartment.

21. The method according to claim 20, wherein the gas partial pressure is the partial pressure of oxygen or carbon dioxide.

22. The method according to claim 20-21, wherein oxygen is supplied to the embryo by diffusion through the stagnant medium in the compartment directly from the atmosphere or from a larger volume of medium in equilibrium with the atmosphere.

23. The method according to any of the claims 21-22, wherein the oxygen partial pressure is measured by an oxygen detector such as an amperometric oxygen detector, a membrane inlet mass spectrometer, a microspectrophotometer, or by optical oxygen sensing.

24. The method according to any of the claims 23, wherein the oxygen partial pressure is measured by optical oxygen sensing, such as by using an immobilized luminophore layer (3) and recording the luminescence.

25. The method according to claim 24, wherein the luminophore comprises Ruthenium(II)-tris-4,7-diphenyl-1,10-phenatroline per chlorate (Rudpp) immobilised in a polystyrene matrix, Ruthenium (II) tris-1,7-diphenyl-1,10-phenanthroline chloride, Ruthenium(II)-tris(bipyridyl) complex, Tris (2,2'-bipyridyl di-chloro-ruthenium)-hexa-hydrate, Ru(bpy), Platinum (II)-octa-ethyl-porphyrin in polystyrene, Platinum (II)-octa-ethyl-porphyrin in poly(methyl-methacrylate), Platinum (II)-octa-ethyl-keto-porphyrin in polystyrene, Platinum (II)-octa-ethyl-keto-porphyrin, Palladium (II)-octa-ethyl-porphyrin in polystyrene, Platinum-1,2-ene-dithiolates (class of compounds).

26. The method according to any of the claims 23-25, wherein the oxygen detector is placed at the bottom of the compartment.

27. The method according to any of the claims 21-26, wherein a gas permeable layer is placed between the embryo and the oxygen detector.

28. The method according to any of the claims 20-27, wherein the response time for the determination of the oxygen partial pressure is less than 10 seconds.

29. The method according to any of the claims 20-28, wherein the determination of gas partial pressure is performed without affecting the growth conditions of the embryo.

30. The method according to any of the claims 21-29, wherein the oxygen partial pressure at the position of the embryo is controlled by adjusting the volume of the compartment (1).

31. The method according to claim 30, wherein the volume is adjusted by the insert (10).

32. The method according to claim 31, wherein the transverse dimensions (A) of the compartment is adjusted by the insert (10).

33. The method according to claim 30, wherein the volume is adjusted by shifting the position of the adjustable bottom (11).

34. The method according to any of the claims 20-33, wherein individual embryos are grown in separate compartments.

35. The method according to claim 34, wherein at least 5 separate compartments are used, particularly at least 10 compartments.

36. The method according to claim 27, wherein the gas permeable layer has a thickness of at least twice the diameter of the embryo.

37. The method according to claim 27, wherein the gas permeable layer has a thickness of at least 100 µm, particularly at least 300 µm, more particularly at least 900 µm.

38. The method according to claim 20, wherein two or more embryos are grown in the same compartment on top of an immobilized luminophore layer.

39. The method according to any of the claims 20-38, wherein the embryos with the most suitable respiration rates are selected for re-implantation.

40. A use of an oxygen partial pressure determination, in stagnant growth medium inside a compartment comprising at least one embryo, for selecting the most viable embryo(s), wherein the oxygen partial pressure at the position of the embryo is dependent on the supply of oxygen from the surroundings to the embryo via an opening by diffusion through the stagnant medium, and said oxygen partial pressure determination is performed without causing any change in growth conditions experienced by the embryo.

41. A method of controlling the oxygen partial pressure experienced by an individual embryo, wherein the said embryo is grown in stagnant medium in a device according to any of the claims 1-20.

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ABSTRACT

The present invention relates to a device and method for non-invasive measurement of the individual respiration rate of at least one embryo and for controlling the oxygen partial pressure experienced by the embryo, in which the embryo is cultured in a gas impermeable compartment having at least one opening, in stagnant medium, and oxygen is supplied from the surroundings to the embryo via the opening by diffusion through the stagnant medium inside the compartment, and determining the respiration rate of the embryo by measuring the oxygen partial pressure at a position inside the compartment.

Figure 1

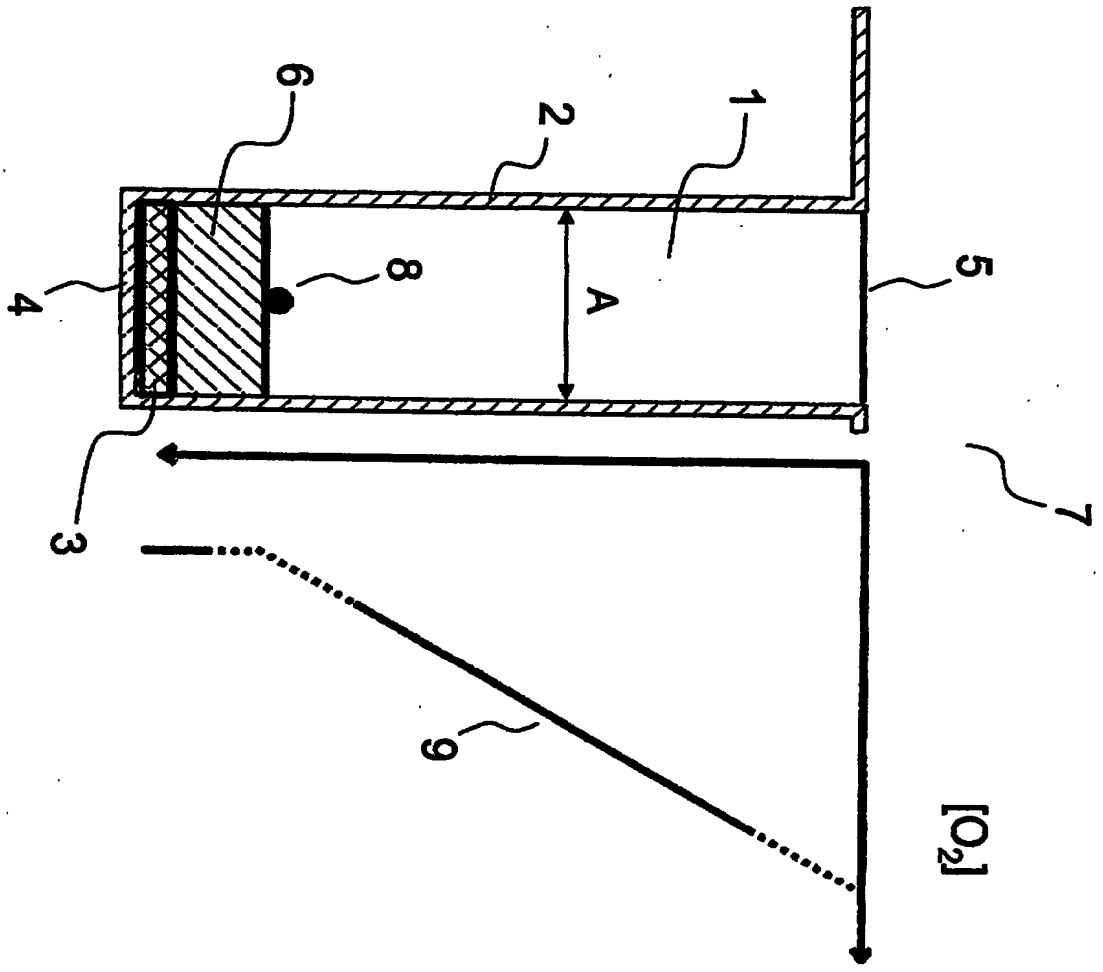

Figure 2

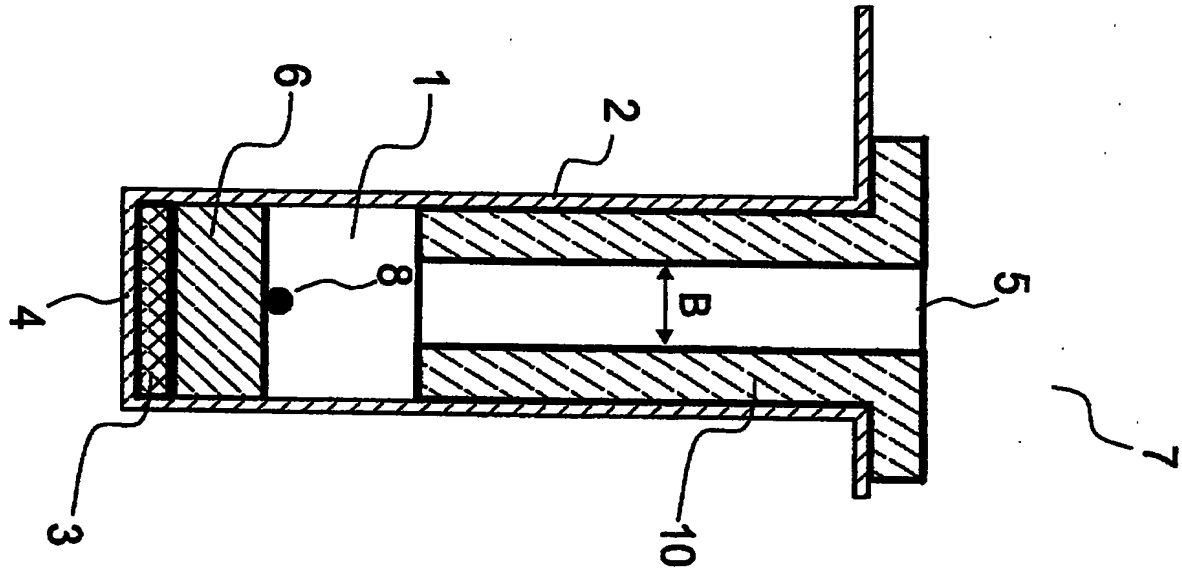

Figure 3

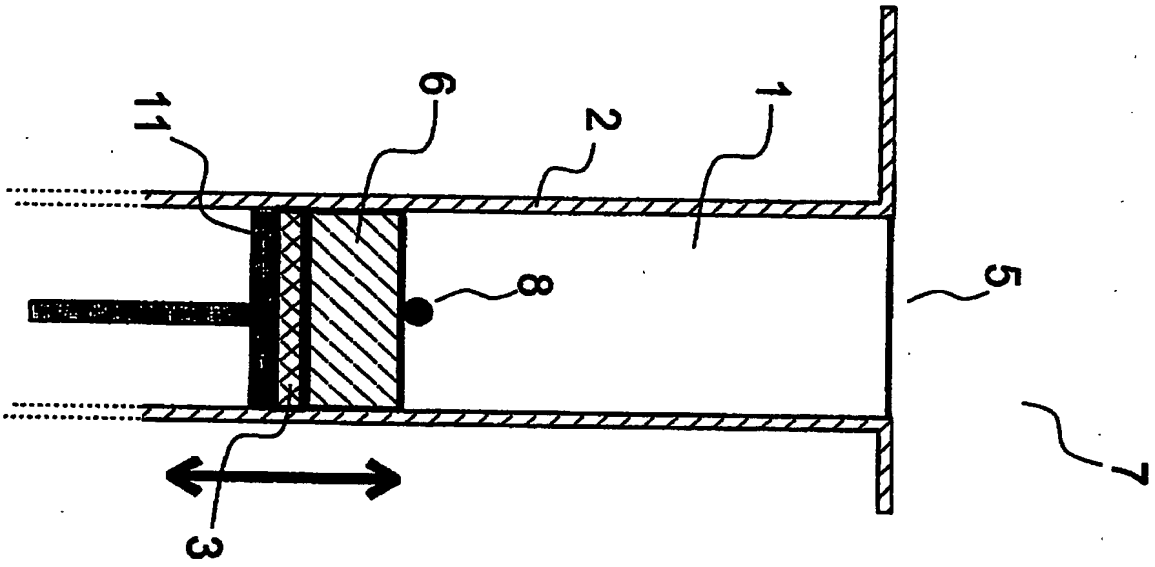

Figure 4

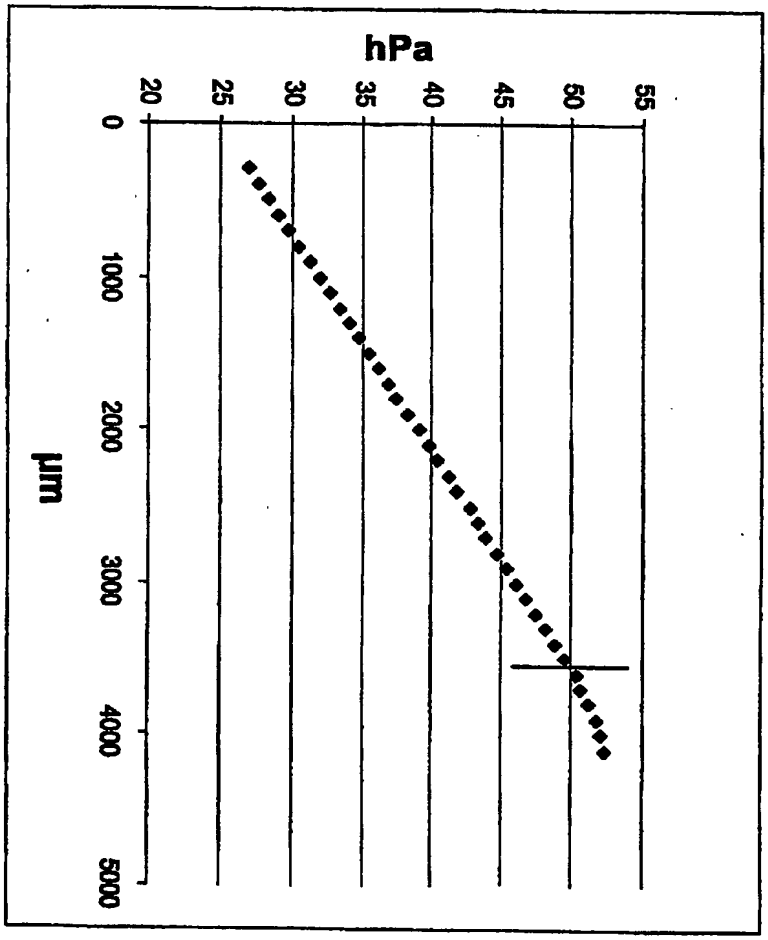

Figure 5

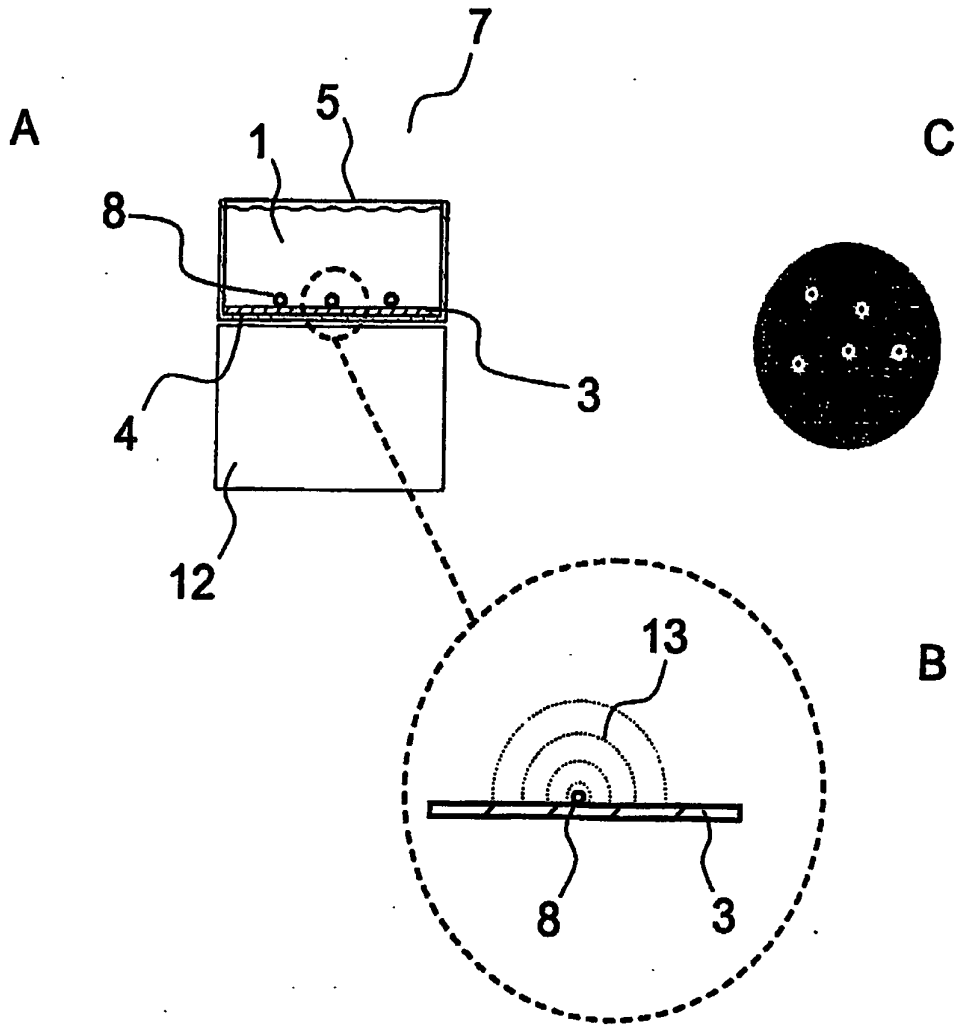

Figure 6